Detection of transmissible viral proventriculitis (TVP) and *Chicken proventricular necrosis virus* (CPNV) in the United Kingdom

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Abstract

Increasing evidence suggests that a new birnavirus, named *Chicken proventricular necrosis virus* (CPNV), is the aetiological agent of transmissible viral proventriculitis (TVP). The present work aimed to explore the possible presence of both TVP and CPNV in the UK. Forty-four chickens showing TVP-compatible gross lesions were classified into 3 groups based on the histological lesions: i) TVP-affected chickens: lymphocytic infiltration and glandular necrosis (n=15); ii) lymphocytic proventriculitis (LP)-affected chickens: lymphocytic infiltration without necrosis (n=18); and iii) without proventriculitis (WP): no lymphocytic infiltration or necrosis (n=11). Nine proventriculi (7 out of 15 corresponding to TVP, and 2 out of 11 corresponding to LP) were positive for CPNV by RT-PCR. These results support the previously suggested idea of CPNV as causative agent of TVP. Moreover, this data shows that CPNV can also be detected in a number of cases with LP, which do not fulfil the histological TVP criteria. Phylogenetic analysis of partial sequences of gene VP1 showed that British CPNV sequences were closer to other European CPNV sequences and might constitute a different lineage from the American CPNV. TVP cases with negative CPNV PCR results may be due to chronic stages of the disease or to the reduced PCR sensitivity on formalin-fixed paraffin embedded tissues. However, involvement of other agents in some of the cases cannot totally be ruled out. As far as the authors are aware, this is the first peer-reviewed report of TVP as well as of CPNV in the UK, and the first exploratory CPNV phylogenetic study.

Keywords: Birnavirus; *Chicken proventricular necrosis virus* (CPNV); transmissible viral proventriculitis (TVP); natural infection; poultry.

Introduction

Transmissible viral proventriculitis (TVP) is an infectious viral disease affecting chickens, which is reported to have a significant economic impact on a global scale (Dormitorio et al., 2007; Guy et al., 2005). Affected chickens display non-specific clinical signs, which may include stunted growth, pallor and the presence of incompletely digested food in the faeces (Goodwin et al., 1996). Flocks affected by TVP typically do not show a significant increase in mortality (Hafner and Guy, 2013). TVP most commonly affects broiler chickens of four to five weeks of age (Bayyari et al., 1995). However, the disease has also been identified in both broiler breeders and layer hens in the age range of nine to twenty weeks (Marusak et al., 2012).

TVP results in enlarged, fragile proventriculi which contain characteristic microscopic lesions (Goodwin et al., 1996; Hafner and Guy, 2013). Chicken flocks are diagnosed with TVP based on the presence of histological lesions in the proventriculus: necrosis of oxynticopeptic cells, lymphocytic infiltration and hyperplastic ductal epithelium which replaces the glandular epithelium (metaplasia) (Goodwin et al., 1996; Hafner and Guy, 2013). No specific control measures or treatments are currently recommended for TVP (Hafner and Guy, 2013).

TVP was first described in the Netherlands almost 40 years ago (Kouwenhoven et al., 1978). Since then, it has been reported in several countries in North-America (Guy et al., 2011b; Noiva et al., 2015), Europe (Grau-Roma et al., 2010; Marguerie et al., 2011) and Asia (Kim et al., 2015). Since the emergence of the disease, there has been considerable discussion as to the aetiological agent responsible. Several viruses have been suggested including: an adenovirus (Kouwenhoven et al., 1978), a reovirus (Jones, 2000), *Infectious bronchitis virus* (IBV) (Yu et al., 2001), *Infectious bursal disease virus* (IBDV) (Huff et al., 2001), and a picornavirus (Kim et al., 2015). Few years ago, a new birnavirus, named *Chicken proventricular necrosis virus* (CPNV), was detected in naturally and experimentally reproduced TVP-affected cases in USA and proposed to be its cause (Guy et al., 2011a; Guy et al., 2011b). CPNV has subsequently

been detected in a few other studies in TVP-affected broiler chickens in France and USA (Marguerie et al., 2011; Noiva et al., 2015).

Despite few cases with proventricular lesions compatible to TVP have previously been reported in the UK[1] (Randall & Reece, 1996), as far as the authors are aware, there is no peer-reviewed report indicating the presence of neither TVP nor CPNV in the United Kingdom (UK). Based on the recent description of both TVP (Grau-Roma et al., 2010; Marguerie et al., 2011) and CPNV (Marguerie et al., 2011) in Europe, the aims of this study were to determine whether TVP and CPNV are present in chickens in the UK, as well as to evaluate their association suggested previously.

Materials and methods

Study design. At the end of 2014, a prospective study was designed based on the collection of samples from chicken post-mortems performed by poultry clinicians in different locations of the UK. Clinicians were asked to send proventricular samples fixed in 10% formalin to the School of Veterinary Medicine and Science (SVMS) at the University of Nottingham when TVP-compatible gross lesions were observed. These lesions included thickening of the proventricular wall, dilation of proventriculus and/or evidence of white spots visible through the proventricular serosa. Clinicians were also asked to report any other relevant abnormalities observed during the post-mortem examination.

In addition, chicken cases received at the Veterinary Pathology Service (VPS) of the SVMS between January 2014 and June 2015 having a diagnosis of 'lymphocytic and/or necrotizing proventriculitis' were included in the study as suspected TVP-affected cases.

[1] VLA (currently APHA) quarterly surveillance report: January-March, Volume 14, No. 1, page 14 (http://www.thepoultrysite.com/articles/1765/uk-poultry-disease-quarterly-surveillance-report-januarymarch-2010).

Histopathology. A complete cross-section on the central area of the proventriculus was performed from each chicken, routinely processed for histology and stained with haematoxylin and eosin. Proventriculi were assessed for the three key histopathological findings which are characteristic of TVP: (i) glandular lymphocytic infiltration; (ii) hyperplastic and metaplastic changes of ductal epithelial cells; and (iii) necrosis of oxynticopeptic cells. These parameters were semi-quantified as follows: - (absence), + (>0 to 10% of the glands affected), ++ (>10 to 50% of the glands affected), +++ (>50% of the glands affected). In addition, the presence of necrosis was also assessed as the percentage of glandular parenchyma affected in the most affected gland, following the same percentages as described above. A mean of the 2 necrosis scores was then calculated, and mean scores between severity levels (e.g. +/++) were rounded up to the higher level. The presence of inflammatory infiltrate within the lamina propria was not taken into consideration, as it is reported to be a frequent finding in healthy birds (Kadhim *et al.*, 2011).

Based on the histopathological results, chickens were allocated a case status as follows: (i) transmissible viral proventriculitis (TVP)-affected chickens: lymphocytic infiltration and necrosis present in the proventriculus; (ii) lymphocytic proventriculitis (LP)-affected chickens: lymphocytic infiltration without necrosis present in the proventriculus; (iii) chickens without proventriculitis (WP): no lymphocytic infiltration or necrosis present in the proventriculus.

RNA extraction and RT-PCR. RNA was extracted from all formalin-fixed paraffin embedded (FFPE) proventriculi and tested subsequently by RT-PCR for CPNV. RNA extraction was done using four 25 μm-sections of each sample. Briefly, the extraction method used incubation with xylol (twice) followed by centrifugation. Pellet re-suspension was performed first with ethanol and second with a digestion buffer containing Proteinase K (Roche, Mannheim, Germany). After overnight incubation at 56°C and centrifugation, supernatant was mixed with TRIzol® Reagent (Invitrogen, 15596-018). Samples were then homogenized with chloroform (Sigma,

C2432) and centrifuged. The transparent phase was discharged and the pellet was mixed with isopropanol (Sigma, I9516). Two more centrifugations followed by addition of cold ethanol were performed. Finally, the pellet was left to dry and received 25 µl of warm RNase-free water.

A RT-PCR procedure was performed to amplify a 171 nucleotide (nt) sequence within the VP1 gene of CPNV using primers and protocols described previously (Guy et al., 2011b). FTA cards with proventricular imprints from positive CPNV cases, kindly provided by Dr. Guerin (National Veterinary School of Toulouse, France), were used as positive controls.

Sequencing of RT-PCR product and phylogenetic studies. The amplified products from the positive CPNV RT-PCR cases were purified using Mini Elute Gel Extraction Kit (Qiagen, Valencia, CA). Sequencing reactions were performed with ABI Prism BigDye Terminator Cycle Sequencing v.31 Ready Reaction (Applied Biosystems, Foster City, CA), and analysed using an ABI Prism model 3730 automated sequencer (Applied Biosystems, Foster City, CA). Positive and negative controls of extraction and amplification were added to each batch of samples tested.

Partial VP1 CPNV sequences obtained from British cases were compared with the sequence of the American CPNV isolate R11/3 (Guy *et al.*, 2011a) available in the Genbank (http://www.ncbi.nlm.nih.gov, accession number HM038436.1), partial sequences obtained from Spanish cases (Costa et al., submitted for publication) and the positive control (FTA card) using MEGA6 (Molecular Evolutionary Genetics Analysis version 6.0) software (Tamura et al., 2013). Sequences were aligned using ClustalW method. A nucleotide distance matrix between sequences was computed to infer phylogenies and a Neighbor-joining (NJ) phylogenetical tree was generated. The partial VP1 CPNV sequences reported in this work have been deposited at GenBank under accession numbers KU933595 to KU933603.

Statistical analyses. Minitab version 17 was used for statistical analyses. Values -, +, ++ and +++ from the histological assessment were converted to 0, 1, 2 and 3, respectively, prior to the analyses. The distribution of variables was assessed using the Ryan-Joiner test. Kruskal-Wallis and Mann-Whitney U tests were used to assess for differences in variables between case statuses for nonparametric data.

Results

Epidemiological data and case status allocation. Forty-four chickens were included in this study (Table 1). Thirty-nine chickens came from the prospective study (chickens with TVP-compatible gross lesions, chickens 1 to 39) and five chickens were received in the VPS and were selected due to the presence of lymphocytic infiltration and/or necrosis of oxynticopeptic cells (suspected TVP-affected cases, chickens 40 to 44). All the chickens were received between April 2014 and June 2015. The farm postcode was provided in 40 out of the 44 chickens, showing that the chickens came from 17 different farms located in England (n=16) and Wales (n=1). The number of chickens received per farm ranged between 1 and 7. All the received chickens were reported to have thickened and/or dilated proventriculus (Figure 1). Most of the received cases corresponded to broiler chickens (42 out of 44), 1 corresponded to a layer hen (chicken 44), while the age of the chicken was not indicated in the remaining case.

Out of the 44 chickens studied, a total of 15 (34%) were classified as TVP-affected chickens, 18 (41%) as LP-affected chickens, and 11 (25%) as chickens without proventriculitis (WP) (Table 1). Excluding the layer hen, the mean±SD age for each case status was: TVP=36±12, LP=24±6 and WP=20±6 days. The only studied layer hen was 38 weeks-old, and was classified within the TVP-affected chickens. In 8 out of the 15 TVP-affected chickens, submitted veterinarians reported abnormal intestinal contents (including orange jejunal contents, intestinal dilation and loose caecal contents).

The TVP-affected chickens came from 9 different farms, all of them located in England. In these farms, the mean±SD number of chickens on a farm was 64,417±56,781, with a range of 20,000 to 203,000 chickens. The vaccination status was available for 5 out of these 9 farms (from were 10 TVP-affected chickens came from, specifically cases 2, 3, 15, 17, 20 to 24 and 41). All of them were vaccinated with a live attenuated IBV vaccine variant strain 4-91, which was combined with the virus strain IB Ma5 in 4 of the farms. In addition, the latter 4 farms also used a live IBDV vaccine containing IBDV strain 228E. No other vaccines were used in these farms. Data on monthly percentage mortality of flocks was available from 4 farms, which ranged from 3.03% to 4.49%.

Histopathology. The histopathological results of each chicken are detailed in Table 1.

The mean necrosis of oxynticopeptic cells score was mild (+) in 8 out of the 15 TVP-affected cases (53%), and moderate (++) in the remaining 7 (47%). Necrotic cells showed hypereosinophilia, fragmentation and karyorrhexis, karyolysis and/or pyknosis, and were usually seen as small clusters within the lumen of dilated proventricular alveoli, often within the edge of the lobule (Figure 2). Collecting ducts (secondary ducts) were often dilated and filled with necrotic debris and sloughed cells. No inclusion bodies were observed in any of the cases.

Thirteen out of the 15 TVP-affected chickens (87%) had severe lymphocytic infiltration, while in the remaining 2 chickens (13%) the lesion was moderate. Lymphocytic infiltration was usually multifocal, located within the interstitium of the proventricular glands (Figure 3). In some cases, lymphocytic cells formed nodular aggregates. Unaffected glands were usually intermingled with affected ones. The median of the lymphocytic infiltration score in the TVP-affected group (3) was significantly higher than in the LP-affected group (1.5) ($p=0.002$).

Although the inflammation in TVP-affected cases was predominantly lymphocytic, few plasma cells, macrophages and occasional heterophils were also present.

Finally, all the 15 TVP-affected chickens (100%) had severe hyperplasia and metaplasia of ductal epithelial cells (Figure 2 and 3), whereas it was present in 11 out of 18 (61%) LP-affected cases and in 2 out of the 11 (18%) chickens within the WP group. The median score for TVP-affected group (3.0) was significantly higher than for LP-affected group (1.5) ($p<0.001$) and for WP (0.0) ($p<0.001$). However, trend only was observed between the median score for LP and WP ($p=0.053$).

CPNV RT-PCR and phylogenetic studies. Nine chickens gave positive results for CPNV RT-PCR in the proventriculus (Table 1). Seven out of these 9 chickens (78%) belonged to the TVP-affected group and the remaining 2 (22%) to the LP-affected group. When looking at each group, 7 out of the 15 TVP-affected cases (47%) and 2 out of the 18 LP-affected chickens (11%) were positive for CPNV RT-PCR. None of the proventriculi from chickens belonging to the WP group gave positive results.

All RT-PCR CPNV positive cases corresponded to broiler chickens. The only analysed layer hen, which was histologically classified as TVP, gave a negative CPNV RT-PCR results.

The VP1 gene of the 9 positive CPNV cases was partially sequenced, including a fragment of 171 nucleotides (nt). Figure 4 shows a phylogenetical tree including these 9 sequences, 1 sequences obtained from the French CPNV RT-PCR positive control, 2 Spanish CPNV sequences and 1 American case (Genbank accession number: HM038436.1). All the 9 British CPNV sequences were closer to each other than to sequences obtained from other countries. Their percentage of similarity was below 90% only when compared with the American and one of the Spanish sequences. Specifically, the cases from the UK showed 99.4-100% nucleotide similarity between the sequences. When compared with the sequences retrieved

from the other countries, the % similarity was 92.4- 92.8%, 88.3-94.2% and 89.5-90.1%, with the French, Spanish and American sequences, respectively (Table 2).

Discussion

Cases of chickens with lymphocytic and necrotising proventriculitis, consistent with TVP, have been reported in the USA (Guy et al., 2011b), South Korea (Kim et al., 2015) and several countries in Europe (Grau-Roma et al., 2010; Kouwenhoven et al., 1978; Marguerie et al., 2011). However, as far as the authors are aware, this is the first peer-reviewed description of TVP as well as of CPNV in the UK.

The mean mortality of broiler flocks in the UK has previously been reported at 4.1% (Dawkins et al., 2004). This percentage is close to the mortality recorded in the TVP-affected farms in this study. These results are therefore in line with earlier studies that state there is no significant increase in mortality in flocks affected by TVP (Hafner and Guy, 2013). All the TVP-affected chickens came from farms located in counties across England. TVP-affected broilers were in the age range of 21 to 49 days old, which is consistent with previous reports of the disease (Bayyari et al., 1995; Hafner and Guy, 2013). The majority of the chickens submitted to the study were Ross 308 broiler chickens, with only 1 case corresponding to a layer hen. There may be 2 reasons for this: (i) TVP affects mainly broiler chickens; (ii) broilers chickens equate to 80% of the chicken post-mortems carried out by the poultry clinicians submitting the samples. The studied layer hen studied was histologically classified as TVP, becoming the second report of this disease in layer hens in peer-reviewed literature (Marusak et al., 2012). Marusak et al. (2012) diagnosed TVP in broiler breeder and commercial layer hens ranging from 9 to 20 weeks of age. Therefore, the hen included here is the oldest chicken reported to be affected by TVP, although it was negative by CPNV RT-PCR.

The detection of CPNV by RT-PCR in almost 50% (7 out of 15) of TVP-affected chickens together with the negative results in all the chickens within the WP group supports the idea that CPNV is the cause of TVP (Guy et al., 2011a; Guy et al., 2011b). All the TVP-affected cases showed moderate to severe lymphocytic infiltrates and severe tubular hyperplasia and metaplasia, which are features of chronicity as demonstrated in experimentally reproduced TVP-affected cases (Guy et al., 2011b). This experimental infection also showed that CPNV was only detectable by RT-PCR from 1 to 14 days post exposure (PE), while the microscopic lesions were present from 5 to 35 days PE (Guy et al., 2011b). Therefore, the TVP cases with negative RT-PCR CPNV in the present study may correspond to chronically CPNV infected chickens, where the virus is not detectable further within the lesions. In addition, the well-known reduced sensitivity of RT-PCR on FFPE tissues compared to fresh tissues might account for a number of these negative RT-PCR results (Lewis et al., 2001). Finally, it can not however be ruled out that other infectious or non-infectious agents, alone or together with CPNV, may be involved in some of the TVP-affected chickens presented here (Huff et al., 2001; Dormitorio et al., 2007; Kim et al., 2015).

It has been reported that proventriculitis can be caused by a number of different factors, including infectious (viruses, bacteria, fungi or parasites), mycotoxins and nutritional factors (Dormitorio et al., 2007). It seems likely that, in this study, the group of LP-affected chickens correspond to a mixture of cases with different aetiologies. Amongst them, a number of LP-affected chickens with negative CPNV RT-PCR results may correspond to chronically affected TVP cases, where the virus is not detectable (Guy et al., 2011b). Interestingly, 11% of LP-affected chickens gave positive results by CPNV RT-PCR. These chickens likely correspond to chronic TVP-affected chickens, where necrosis of oxynticopeptic cells is not observed, but where the virus is still detectable. This finding indicates that TVP should still be suspected in cases of LP without glandular necrosis and that the CPNV RT-PCR can have diagnostic value

in these cases. It is worth mentioning that areas of necrosis may have been present within other non-examined areas of the proventriculi, since this study was performed on a single complete cross-section of each proventriculus.

The % of nucleotide similarities showed the highest value similarity when comparing the sequences obtained from within the UK. Moreover, the phylogenetical tree of nucleotide sequences suggested that the UK CPNV sequences may be more similar to the other European sequences than to the American sequence. These geographical variations may be due to mutations as a result of different selection pressures between countries and continents, as previously suggested for other virus such as IBDV (Jackwood and Sommer-Wagner, 2007). Although the current study included short nucleotide sequences (171 nt) within the VP1 gene, it must be acknowledged that this gene encodes for the RNA-dependent RNA-polymerase, which is known to be a well-conserved gene in cellular organisms as well as in viruses (Pan et al., 2007). For this reason, based on the results obtained, it could be hypothesized that two distinct lineages of CPNV, i.e. European and American, are present in the two continents. It would, however, be necessary to perform larger studies, increasing the number of sequences and their length, to try to confirm these initial observations

In conclusion, this study identified the presence of TVP amongst chicken populations in the UK. Results indicate that CPNV can often be detected within the proventriculus of TVP-affected chickens, and in a number of chickens with LP. Moreover, preliminary phylogenetic studies on partial CPNV sequences indicate that European and American chicken populations may have 2 different CPNV genetic lineages. Additional investigations, encompassing larger sample sizes, are needed in order to determine the incidence and prevalence of TVP in the UK and globally.

Acknowledgements

The authors would like to thank A. McDaniel, W. Garton, C. Blake-Dyke, M. Phelps and C. Lopez from Minster Veterinary Practice who kindly provided the proventricular samples. We also thank Dr. Guerin, from the National Veterinary School of Toulouse (France) for kindly providing CPNV positive controls and Rosa Maria Valle and Mónica Pérez (CReSA, IRTA-UAB) and Alan Lasslett (UoN) for technical support. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007-2013) under REA grant agreement No PCOFUND-GA-2012-600181.

References

Bayyari, G.R., Huff, W.E., Balog, J.M., Rath, N.C., Beasley, J.N. (1995). Experimental reproduction of proventriculitis using homogenates of proventricular tissue. *Poultry Science*, 74, 1799-1809.

Dawkins, M.S., Donnelly, C.A., Jones, T.A. (2004). Chicken welfare is influenced more by housing conditions than by stocking density. *Nature*, 427, 342-344.

Dormitorio, T.V., Giambrone, J.J., Hoerr, F.J. (2007). Transmissible proventriculitis in broilers. *Avian Pathology*, 36, 87-91.

Goodwin, M.A., Hafner, S., Bounous, D.I., Latimer, K.S., Player, E.C., Niagro, F.D., Campagnoli, R.P., Brown, J. (1996). Viral proventriculitis in chickens. *Avian Pathology*, 25, 369-379.

Grau-Roma, L., Marco, A., Martinez, J., Chaves, A., Dolz, R., Majo, N. (2010). Infectious bursal disease-like virus in cases of transmissible viral proventriculitis. *Veterinary Record*, 167, 836.

310 Guy, J.S., Barnes, H.J., Smith, L., Owen, R., Fuller, F.J. (2005). Partial characterization of an
311 adenovirus-like virus isolated from broiler chickens with transmissible viral
312 proventriculitis. *Avian Diseases*, 49, 344-351.
313 Guy, J.S., West, A.M., Fuller, F.J. (2011a). Physical and genomic characteristics identify
314 chicken proventricular necrosis virus (R11/3 virus) as a novel birnavirus. *Avian*
315 *Diseases*, 55, 2-7.
316 Guy, J.S., West, M.A., Fuller, F.J., Marusak, R.A., Shivaprasad, H.L., Davis, J.L., Fletcher,
317 O.J. (2011b). Detection of chicken proventricular necrosis virus (R11/3 virus) in
318 experimental and naturally occurring cases of transmissible viral proventriculitis with
319 the use of a reverse transcriptase-PCR procedure. *Avian Diseases,* 55, 70-75.
320 Hafner, S., Guy, J.S. (2013). Proventriculitis and proventricular dilatation of broiler chickens.
321 In: Swayne, D. E., Glisson, J. R., McDouglald, L. R., Nolan, L. K., Suarez, D. L. and
322 Nair, V. L. *Diseases of Poultry* 13th edn (pp. 1328-1332). Wiley-Blackwell Publishing,
323 Ames, USA.
324 Huff, G.R., Zheng, Q., Newberry, L.A., Huff, W.E., Balog, J.M., Rath, N.C., Kim, K.S.,
325 Martin, E.M., Goeke, S.C., Skeeles, J.K. (2001). Viral and bacterial agents associated
326 with experimental transmission of infectious proventriculitis of broiler chickens. *Avian*
327 *Diseases*, 45, 828-843.
328 Jackwood, D.J., Sommer-Wagner, S. (2007). Genetic characteristics of infectious bursal
329 disease viruses from four continents. *Virology*, 365, 369-375.
330 Jones, R.C. (2000). Avian reovirus infections. Rev. Sci. Tech. 19, 614-625.
331 Kim, H.-R., Yoon, S.-J., Lee, H.-S., Kwon, Y.-K. (2015). Identification of a picornavirus from
332 chickens with transmissible viral proventriculitis using metagenomic analysis. *Archives*
333 *of Virology*, 160, 701-709.

Kouwenhoven, B., Davelaar, F.G., Van Walsum, J. (1978). Infectious proventriculitis causing runting in broilers. *Avian Pathology*, 7, 183-187.

Kadhim, K.K., Zuki, A.B., Noordin, M.M. & Babjee, S.M. (2011). Histomorphology of the stomach, proventriculus and ventriculus of the red jungle fowl. *Anatomia Histologia Embryologia*, 40, 226-233.

Lewis, F., Maughan, N.J., Smith, V., Hillan, K., Quirke, P. (2001). Unlocking the archive--gene expression in paraffin-embedded tissue. *The Journal of Pathology*, 195, 66-71.

Marguerie, J., Leon, O., Albaric, O., Guy, J.S., Guerin, J.-L. (2011). Birnavirus-associated proventriculitis in French broiler chickens. *Veterinary Record*, 169, 394-396.

Marusak, R.A., West, M.A., Davis, J.F., Fletcher, O.J., Guy, J.S. (2012). Transmissible viral proventriculitis identified in broiler breeder and layer hens. *Avian Diseases*, 56, 757-759.

Noiva, R., Guy, J.S., Hauck, R., Shivaprasad, H.L. (2015). Runting Stunting Syndrome Associated with Transmissible Viral Proventriculitis in Broiler Chickens. *Avian Diseases*, 59, 384-387.

Pan, J., Vakharia, V.N., Tao, Y.J. (2007). The structure of a birnavirus polymerase reveals a distinct active site topology. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 7385-7390.

Randall, C.R. & Reece, R.L. (1996). Color atlas of avian histopathology. Ed. Mosby-Wolfe, imprint Times Mirror International Publishers Limited, London, UK, p. 57.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30, 2725-2729.

Yu, L., Jiang, Y., Low, S., Wang, Z., Nam, S.J., Liu, W., Kwangac, J. (2001). Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. *Avian Diseases*, 45, 416-424.

Tables

Table 1. Microscopic proventricular lesion scores[a], RT-PCR results and case status[b] for each chicken. Genbank accession numbers for positive *Chicken proventricular necrosis virus* (CPNV) RT-PCR cases are detailed.

	Microscopic proventricular lesion scores						
Chicken	**Mean necrosis score[c]**	**Interstitial lymphocytic infiltration**	**Ductal epithelial hyperplasia and metaplasia**	**Case Status**	**CPNV RT-PCR**	**Identifier**	**Accession number**
1	-	-	-	WP	Negative		
2	+	+++	+++	TVP	Negative		
3	+	+++	+++	TVP	Negative		
4	-	-	-	WP	Negative		
5	-	-	-	WP	Negative		
6	-	+	-	LP	Negative		
7	-	+++	+++	LP	Negative		
8	-	+	-	LP	Negative		
9	-	++	++	LP	Negative		
10	-	+	-	LP	Negative		
11	-	-	-	WP	Negative		
12	-	-	-	WP	Negative		
13	-	+	-	LP	Negative		
14	-	+++	++	LP	Negative		
15	++	+++	+++	TVP	**Positive**	CPNV-UK-1	KU933597
16	-	++	++	LP	**Positive**	CPNV-UK-2	KU933598
17	++	+++	+++	TVP	Negative		
18	-	+	++	LP	Negative		
19	-	-	++	WP	Negative		
20	++	+++	+++	TVP	Negative		
21	+	+++	+++	TVP	**Positive**	CPNV-UK-3	KU933599
22	++	++	+++	TVP	**Positive**	CPNV-UK-4	KU933600
23	++	+++	+++	TVP	**Positive**	CPNV-UK-5	KU933601
24	++	+++	+++	TVP	**Positive**	CPNV-UK-6	KU933602
25	+	++	+++	TVP	Negative		
26	+	+++	+++	TVP	Negative		
27	+	+++	+++	TVP	Negative		
28	-	++	-	LP	Negative		
29	-	+++	++	LP	Negative		
30	-	-	-	WP	Negative		
31	-	+	++	LP	Negative		
32	-	-	-	WP	Negative		
33	-	+	+	LP	Negative		
34	-	-	++	WP	Negative		
35	-	+	-	LP	Negative		
36	-	-	-	WP	Negative		
37	-	+	+	LP	Negative		
38	-	-	-	WP	Negative		
39	-	++	-	LP	Negative		
40	-	+++	+++	LP	Negative		

41	+	+++	+++	TVP	**Positive**	CPNV-UK-7	KU933603
42	-	+++	+++	LP	**Positive**	CPNV-UK-8	KU933595
43	++	+++	+++	TVP	**Positive**	CPNV-UK-9	KU933596
44	+	+++	+++	TVP	Negative		

[a]-: absence; +: >0 to 10% of the glands affected; ++: >10 to 50% of the glands affected, +++: >50% of the glands affected

[b]TVP: transmissible viral proventriculitis; LP: lymphocytic proventriculitis; WP: without proventriculitis.

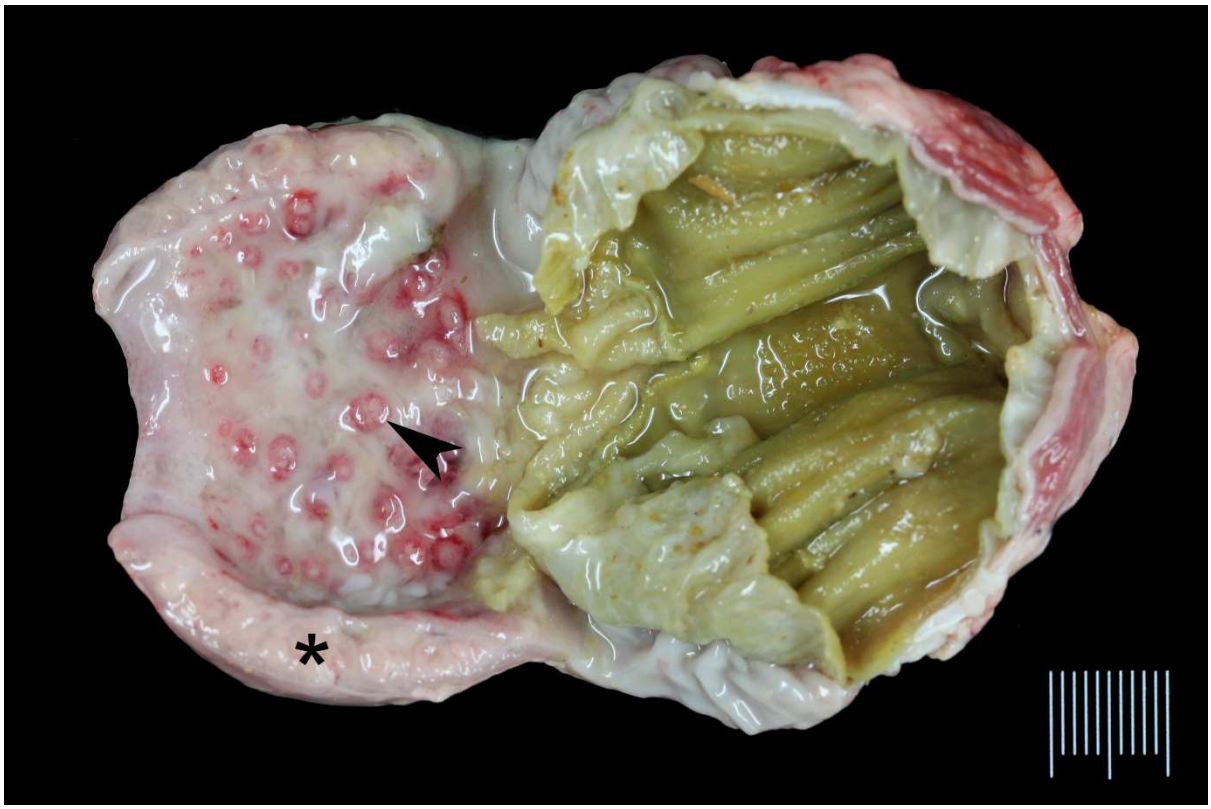
[c]Mean necrosis score is the mean combined score of necrosis of oxynticopeptic cells in all glands and necrosis of glandular parenchyma in the most affected gland.

Table 2. Percentage of homology between the studied *Chicken proventricular necrosis virus* (CPNV) partial VP1 sequences. Sequences included are American (CPNV-USA, n=1), French (CPNV-Fr, n=1), Spanish (CPNV-Sp, n=2) and British (CPNV-UK, n=9).

	CPNV-USA-1	**CPNV-Fr-1**	**CPNV-Sp-2**	**CPNV-Sp-1**	**CPNV-UK-8**	**CPNV-UK-9**	**CPNV-UK-1**	**CPNV-UK-2**	**CPNV-UK-3**	**CPNV-UK-4**	**CPNV-UK-5**	**CPNV-UK-6**	**CPNV-UK-7**
CPNV-USA-1	100.00	90.06	89.47	91.81	89.47	89.47	90.06	90.06	90.06	90.06	90.06	90.06	90.06
CPNV-Fr-1	90.06	100.00	94.15	92.40	92.40	92.40	92.98	92.98	92.98	92.98	92.98	92.98	92.98
CPNV-Sp-2	89.47	94.15	100.00	94.74	93.57	93.57	94.15	94.15	94.15	94.15	94.15	94.15	94.15
CPNV-Sp-1	91.81	92.40	94.74	100.00	88.30	88.30	88.89	88.89	88.89	88.89	88.89	88.89	88.89
CPNV-UK-8	89.47	92.40	93.57	88.30	100.00	100.00	99.42	99.42	99.42	99.42	99.42	99.42	99.42
CPNV-UK-9	89.47	92.40	93.57	88.30	100.00	100.00	99.42	99.42	99.42	99.42	99.42	99.42	99.42
CPNV-UK-1	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CPNV-UK-2	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CPNV-UK-3	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CPNV-UK-4	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CPNV-UK-5	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CPNV-UK-6	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CPNV-UK-7	90.06	92.98	94.15	88.89	99.42	99.42	100.00	100.00	100.00	100.00	100.00	100.00	100.00

401 **Figures**

402



403

404 **Figure 1.** Proventriclulus and gizzard, broiler chicken, chicken 22, Transmissible viral
405 proventriculitis (TVP)-affected case. The proventricular wall is severely and diffusely
406 thickened. Multifocal and small (up to 0.5 cm) circular areas of congestion and haemorrhage
407 are present within the proventricular mucosa generating a pattern that highlights the
408 proventricular mucosal papillae (arrowhead). The proventricular wall shows prominent
409 glandular lobules (asterisk). Gizzard shows no gross lesions.

410

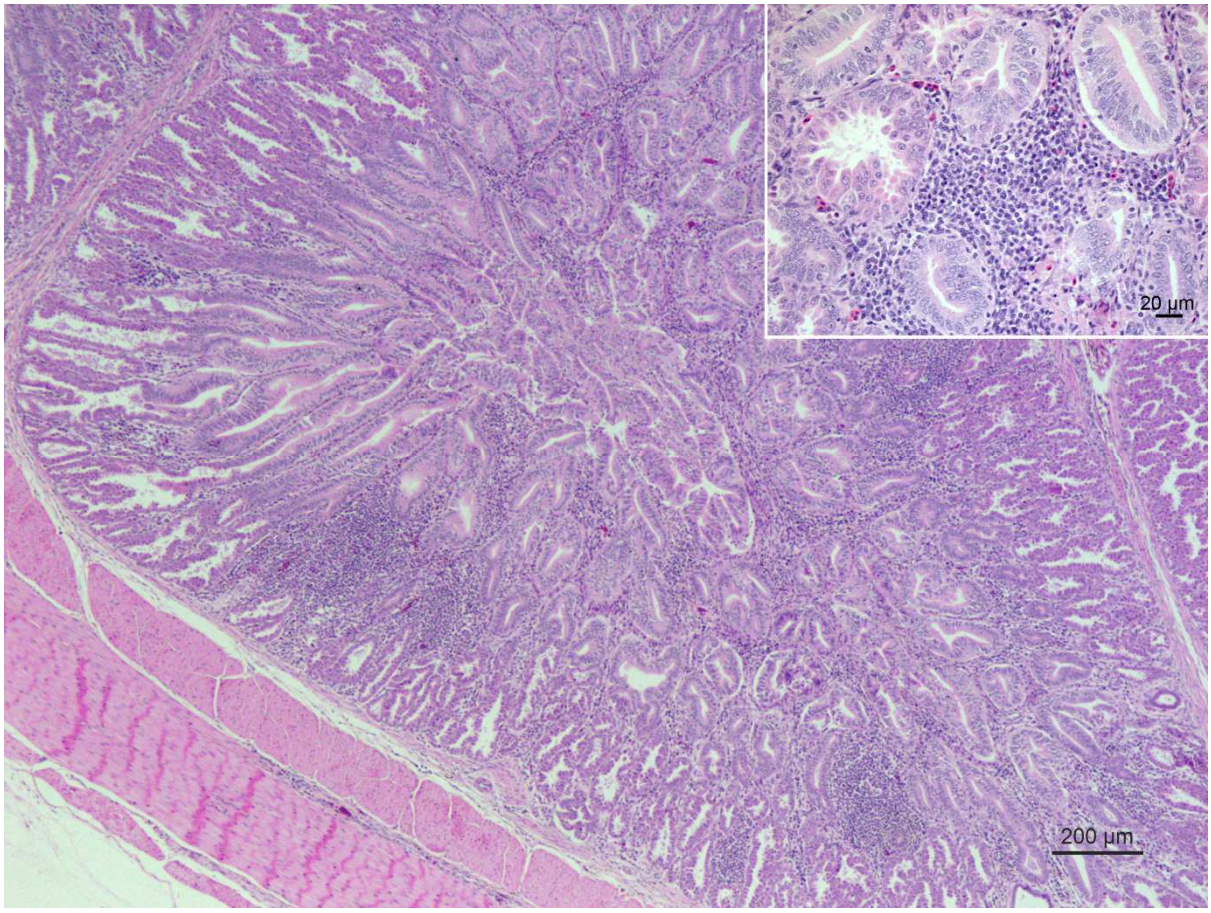


Figure 2. Proventriculus, broiler chicken, chicken 17. Transmissible viral proventriculitis (TVP)-affected case. Photomicrograph showing severe proventricular interstitial lymphocytic infiltration and moderate replacement of glandular epithelium by hyperplastic ductal epithelium (ductal epithelial metaplasia). Inset: Higher magnification of the same proventriculus, showing the predominantly lymphocytic interstitial infiltration as well as the tubular epithelial hyperplasia and metaplasia. Haematoxylin and eosin.

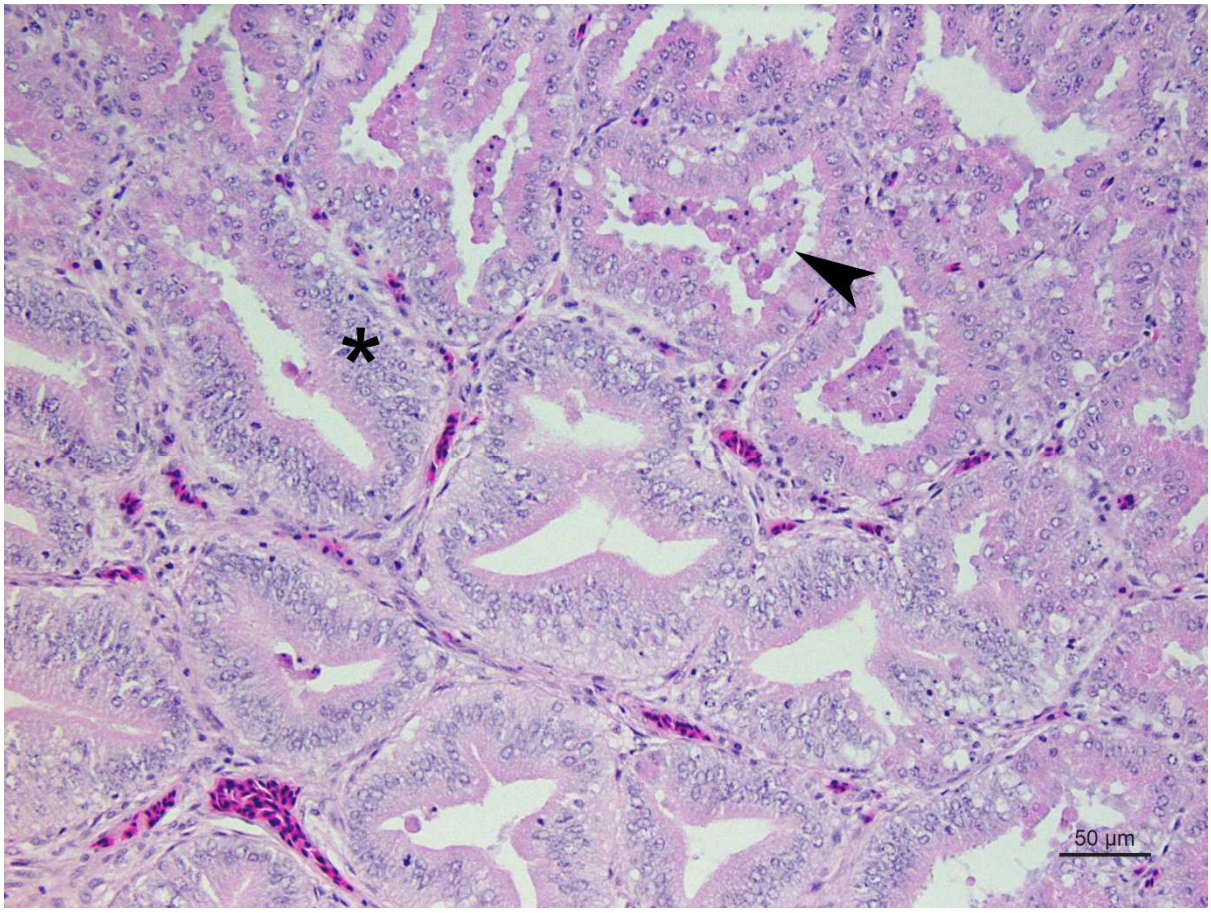


Figure 3. Proventriculus, broiler chicken, chicken 20. Transmissible viral proventriculitis (TVP)-affected case. Photomicropraph showing multifocal aggregates of necrotic cells (arrowhead) within the lumen of proventriculuar alveoli located at the periphery of a proventricular lobule. Areas with replacement of glandular epithelium by hyperplastic ductal epithelium (ductal epithelial metaplasia) are also present (asterisk). Haematoxylin and eosin.

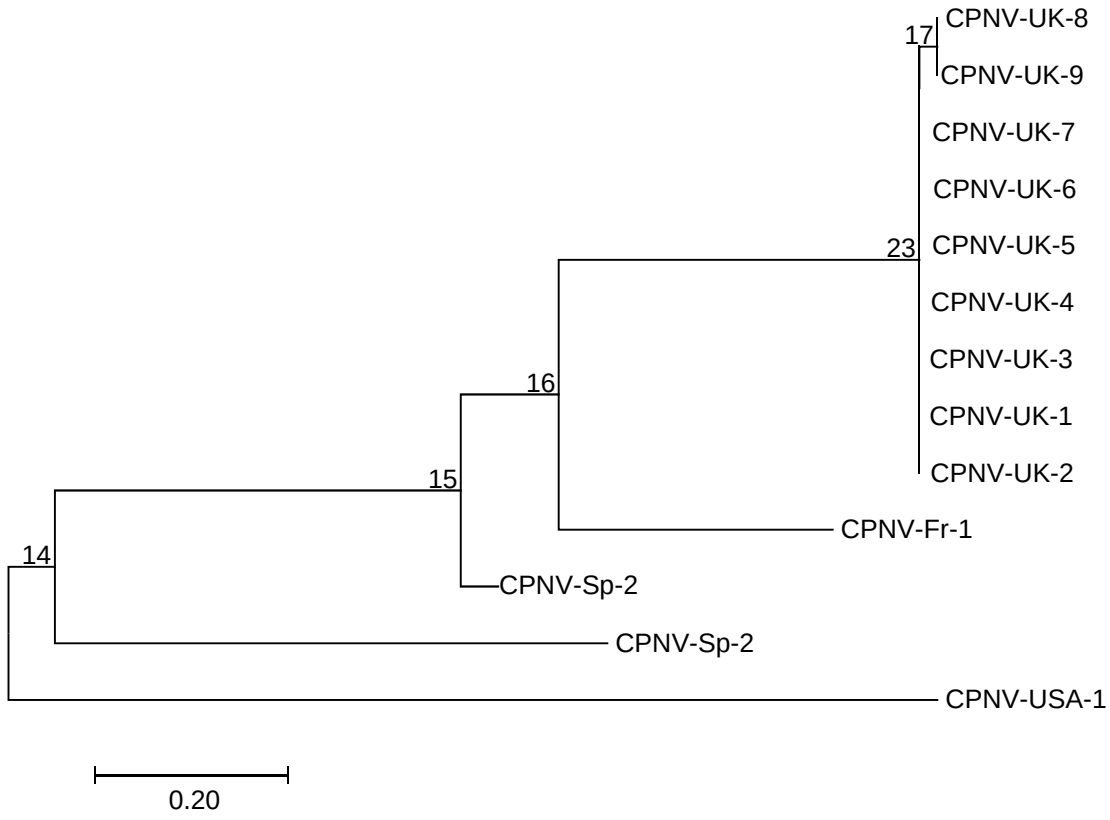


Figure 4. Phylogenetic tree based on the NJ method for 13 partial (171 nucleotides) VP1 CPNV sequences. Sequences originate from 4 different countries: USA (CPNV-USA-1), France (CPNV-Fr-1), Spain (CPNV-Sp-1 and 2), and UK (CPNV-UK-1 to 9). Numbers along the branches refer to the percentages of confidence.